

## Research Article

# Safety Testing of Veterinary Vaccines Using Magnetic Resonance Imaging in Pigs

Maren Bernau<sup>1</sup>, Prisca V. Kremer<sup>1,2</sup>, Elke Pappenberger<sup>1</sup>, Lena S. Kreuzer<sup>1</sup>, Klaus Cussler<sup>3</sup>, Andreas Hoffmann<sup>3</sup> and Armin M. Scholz<sup>1</sup>

<sup>1</sup>Livestock Center Oberschleissheim, Veterinary Faculty of the Ludwig-Maximilians-University Munich, Germany;

<sup>2</sup>University of Applied Sciences, Weihenstephan-Triesdorf, Germany; <sup>3</sup>Paul-Ehrlich-Institut, Federal Institute for Vaccines and Biomedicines, Langen, Germany

### Summary

Safety testing of veterinary vaccines requires the use of a large number of animals to investigate possible local and systemic reactions. This includes, among others, the pathological examination of the injection site in frequent intervals. This examination requires a selected killing of animals in frequent intervals. To reduce the number of animals needed for this kind of safety testing, magnetic resonance imaging (MRI) was used to detect and quantify possible local reactions after vaccination *in vivo*. Sixty-four pigs were divided into four experimental groups ( $n = 16$ ); two groups consisting of 12-week-old pigs and two of 6-month-old pigs at vaccination day. The pigs were vaccinated with four licensed products (each group receiving one vaccine) and examined up to 6 times using MRI during a period of 5 weeks. The MR images were evaluated semi-automatically, comparing the volumes of altered signal intensities on the vaccination side (VS) with the volumes of the signal intensities on the control side (CS). A paired *t*-test was used to identify significant differences ( $p < 0.05$ ) between VS and CS. The results show that MRI allows a 3D-quantification of the extent of local reactions *in vivo* by scanning the same live animals at several time points after vaccination. MRI is a suitable alternative method for non-invasive safety testing of injectable medicines and can therefore be applied to reduce animal numbers used for safety testing purposes.

Keywords: safety testing, magnetic resonance imaging, pigs, veterinary vaccines

## 1 Introduction

Safety testing in animals is an important part of the licensing procedure for veterinary vaccines required by the *European Pharmacopoeia* and other legal regulations, such as Directive 2001/82/EC (EDQM, 2008; EC, 2001). For these regulatory tests a large number of animals is needed, because every target animal species and category, for which the use of a medicinal product is intended, has to be tested. Currently, some animals have to be euthanized in frequent intervals after vaccination in order to perform a pathologic examination of the vaccination site (EC, 2001). Alternative approaches are needed to reduce animal numbers used for regulatory purposes as is demanded by the World Health Organization (WHO). The WHO demanded minimization of the use of animals and pushed forward the use of alternative methods which reduce, refine

or replace animal testing, i.e. the 3R principle (Stokes et al., 2011; Russel and Burch, 1959).

This study was conducted in order to minimize the number of animals used in safety testing of veterinary vaccines according to the 3R principle. Magnetic resonance imaging (MRI) was investigated as a possible alternative method to detect and to evaluate vaccination-induced local reactions at the injection site in the living animal at several time points after vaccination. In human medicine imaging methods like MRI, computer tomography (CT) and ultrasound (US) are approved methods for clinical studies of muscular disorders like inflammation, denervation, mass lesions and their follow-up (Kuo and Carrino, 2007; Reimers, 1990; Schedel et al., 1992; Walker, 2008). Because of its higher sensitivity, a better contrast resolution and the use of non-ionizing radiation, MRI became the method of choice for soft tissue imaging (Fisher et

Received July 7, 2014;  
accepted November 5, 2014;  
Epub December 3, 2014;  
<http://dx.doi.org/10.14573/altex.1407071>



This is an Open Access article distributed under the terms of the Creative Commons Attribution 4.0 International license (<http://creativecommons.org/licenses/by/4.0/>), which permits unrestricted use, distribution and reproduction in any medium, provided the original work is appropriately cited.



al., 1986; Lovitt et al., 2006; Nägele and Hahn, 1990; Tantt and Sepponen, 1996).

The body of a patient consists mainly of muscle, fat, connective tissue and bone. All tissues are mainly built up on protons ( $^1\text{H}^+$  ion). The signal of an MR image is based on the proton distribution, density, relaxation time after a radio frequency impulse and spin “behavior” inside the patient’s body (Pipe, 1999; Hodgson, 2010). The grey scale of a tissue in MR images depends on the intensity of the signal and therefore on intrinsic properties, which change depending on the tissue condition (Hodgson, 2010; Tantt and Sepponen, 1996). Muscle signal intensity can be affected by various conditions such as trauma, infection or inflammation. These abnormalities effect an alteration in tissue parameters and cause differences in signal intensity in the image, as well as in muscle size and profile (Lovitt et al., 2006; May et al., 2000; Schrank et al., 2005). The ability to detect these alterations makes MRI a useful tool in diagnosis and follow-up of musculoskeletal diseases in humans (Lovitt et al., 2006; Messineo et al., 1998).

For clinical safety studies in veterinary medicine the use of MRI is in most cases limited to pet animals. Investigations using farm animals are rare. This study utilized MRI in pigs to investigate vaccination-induced local reactions. The repeated monitoring of animals *in vivo* by MRI, resulting in well-documented clinical data, should allow a reduction in the number of animals needed to perform the safety testing for veterinary medicines.

## 2 Animals and methods

### *Animals, management and testing scheme*

The study was conducted at the Livestock Center Oberschleissheim of the Veterinary Faculty of the Ludwig-Maximilians-University in Munich. For this study a total of 64 pigs were used. The number of animals and the experimental set-up, including housing, keeping and feeding, was conducted in accordance with the District Government of Upper Bavaria (registration number: 55.2-1-54-2532-138-11) and were in compliance with local and national guidelines and reported in detail according to the ARRIVE guidelines (Kilkenny et al., 2010). The animals were kept according to the national animal welfare law (Germany, 2013) and the German Animal Welfare Regulation for Livestock Housing (German Federal Ministry for Food, Agriculture and Consumer Protection, 2009). All animals were crossbred offspring from Piétrain sires and German Landrace

sows, randomly divided into experimental groups as outlined in Table 1. No separate randomization was performed, because each pig was control and treatment animal at the same time. Only healthy pigs originating from a crossbred litter entered the experiment.

The pigs were born and raised at the Livestock Center. The animals grew up and were treated like all other pigs. At an age of four weeks, piglets were weaned and moved to a nursery deck where they were housed in groups of 15-22 animals (0.65-0.75 m<sup>2</sup> per animal). All boxes were enriched with balls and metal chains, which can be moved and changed as required in the German Animal Welfare Regulation for Livestock Housing (German Federal Ministry for Food, Agriculture and Consumer Protection, 2009) to satisfy their exploration drive. At a body weight of 25-30 kg, the pigs were moved to an outdoor-climate barn and housed in group pens combined of deep straw bedding in the rest area and concrete slatted flooring in the feeding area, providing an individual space of > 0.8 m<sup>2</sup>. All pigs were fed with a specific diet (11-13 MJ/kg of ME age-related) twice a day.

Three weeks before the animals reached the specific age for vaccination all pigs were marked with a tattoo needle, creating a circle (diameter 0.3 cm) on the neck skin behind the left ear. This was performed in order to mark the injection point permanently for the whole examination period (see Fig. 1). One day before vaccination, the pigs were moved into a barn nearby the MRI unit. Inside this facility, the pigs were housed in groups of eight pigs in a 12 m<sup>2</sup> pen with concrete flooring and straw bedding. The pigs were fed only once (morning feeding) on the day before scanning and received no food on the examination day in order to prepare a secure anesthesia and avoid vomiting and swallowing during general anesthesia. Access to water was given *ad libitum*.

All pigs were vaccinated into the middle of the tattoo circle (day 0). The groups differed regarding their age at vaccination day (group I + II: 12 weeks old; group III + IV: six months old) and the vaccines used. All vaccines differed in their composition such as antigens, adjuvants, preservatives and other additives (see Tab. 2). All animals were test and drug naïve and had not been vaccinated with the used antigen before. Vaccines were administered by using specific needles according to the age of the pig (group I: 16G x 3/4, Ideal Instruments, Neogen; group II: 18G x 1, Easy Lance, WDT; group III + IV: 17G x 2, Fine-Ject, Henke Hass Wolf).

Before vaccination the health status of each animal was checked. After vaccination (day 0), each animal was scanned

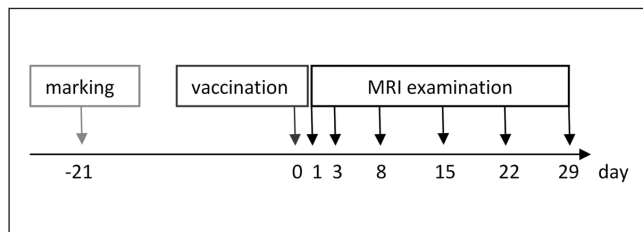
**Tab. 1: Description of the animals in each experimental group**

group	number of animals	gender	age* (days)	weight* (kg)
I	16	male	89 ± 1.02	33.1 ± 4.19
II	16	male	86.13 ± 0.34	29.38 ± 3.66
III	16	female	172 ± 0.45	76 ± 8.22
IV	16	female	171.56 ± 1.21	87 ± 11.61

\*on first day of study (day 1)

**Tab. 2: Vaccine ingredients, injection type and injection volume for each group**

group	age at vaccination day	ingredients of the inactivated product (antigen, adjuvant & preservative)	injection type and volume
I	12 weeks	Gonadotropin releasing factor analogon, diethylaminoethyl-dextran, thiomersal	subcutaneous, 2 ml
II	12 weeks	Erysipelothrix rhusiopathiae, aluminium hydroxide, thiomersal	subcutaneous, 2 ml
III	6 months	Parvovirus + Erysipelothrix rhusiopathiae I, aluminium hydroxide, thiomersal	intramuscular, 2 ml
IV	6 months	Parvovirus + Erysipelothrix rhusiopathiae II DL- $\alpha$ -Tocopherolacetate, formaldehyde	intramuscular, 2 ml


**Fig. 1: Sequence of the procedures**

using MRI on days 1, 3, 8, 15, 22 and 29 (see Fig. 1; not all groups were examined on all examination days). The examination procedure was stopped when no reaction could be observed any longer at the vaccination site. This resulted in different examination periods for the experimental animals. The pigs were anaesthetized before MRI examination using an intramuscular injection of azaperone (2 mg/kg body weight) and ketamine (20–40 mg/kg body weight). As all pigs were vaccinated in the neck as recommended, the anesthetics and all necessary injections before day 0 were injected into the muscles of the hind leg (*Musculus semimembranosus*, *Musculus semitendinosus*) to avoid artifacts in the neck region. The health status of each pig was checked before injection of anesthetics.

#### Sample size

Each experimental group reported here consisted of 16 animals. For sample size calculation the GPower software package 3.1 (Faul et al., 2007, 2009) with an assumed power of 0.8 and an effect size of 0.66, which correlates with a large effect size, was used. The sample size calculation for a matched paired t-test resulted in  $n = 16$ .

#### Magnetic resonance imaging

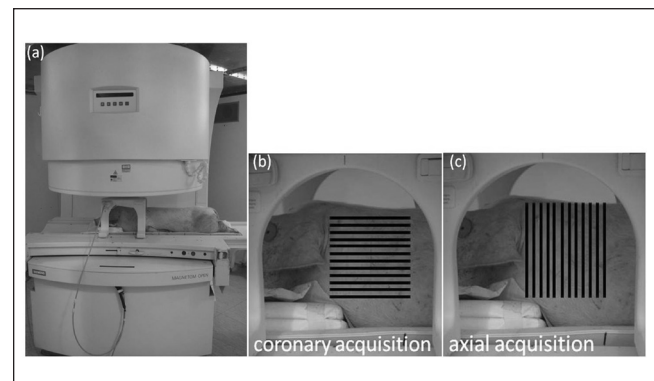
An open low-field MRI system (Siemens Magnetom Open; 0.2 Tesla magnetic field strength) was used for data acquisition. For the detection of muscle abnormalities, like hematoma, edema or fatty infiltration, different MR imaging procedures can be used, like different T1-weighted and T2-weighted image sequences (see Tab. 3). This depends on different relaxation times: transversal (T1) and longitudinal (T2) relaxa-

tion time (for further information see Pipe, 1999 or Hodgson, 2010). Depending on the used sequence weighting, different pathologic conditions can be visualized and result in areas (volumes) with increased signal intensity (Lovitt et al., 2006; May et al., 2000; Schrank et al., 2005). For this study, T1- and T2- weighted spin echo sequences with two directions of acquisition (axial and coronary; only results for the coronary acquisition are displayed) were applied (see Tab. 3 and Fig. 2) in order to illustrate a wide spectrum of pathologic conditions.

The following protocol was used for the MRI examination:

1. T1-weighted spin echo sequence with coronary acquisition (T1<sub>c</sub>)
2. T2-weighted spin echo sequence with the same coronary acquisition as T1<sub>c</sub> (T2<sub>c</sub>)
3. T1-weighted spin echo sequence with axial acquisition (T1<sub>a</sub>) (T1 = T1-weighted; T2 = T2-weighted; c = coronary acquisition; a = axial acquisition)

During the MRI examination the pigs were bedded in a prone position with their front limbs flexed and hind limbs extended. The small body coil (for the 12 week old pigs) and the large


**Fig. 2: Demonstration of the different acquisition directions**

The black lines (inside b & c) demonstrate the acquisition directions (coronary and axial). Image (a) shows a pig inside the Siemens Magnetom Open, positioned for examination of the pigs' neck. The black lines inside image (b) demonstrate the coronary image acquisition. The black lines inside image (c) demonstrate axial image acquisition.

**Tab. 3: MRI parameters for the examination of vaccination-induced local reactions in pigs**

For the different age groups (group I & II: 12 weeks old; group III & IV: 6 months old) different MRI protocols had to be used because of the different animal sizes. T1wSE = T1-weighted spin echo sequence with coronary and axial acquisition. T2wSE = T2-weighted spin echo sequence with coronary acquisition

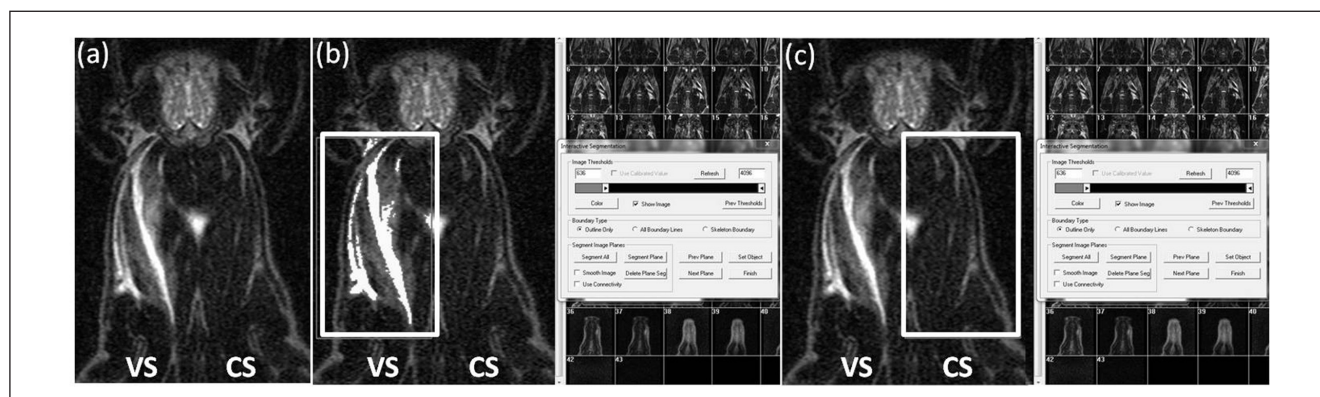
	group I & II		group III & IV	
	T1wSE	T2wSE	T1wSE	T2wSE
pixel size (mm)	1.67 x 0.90	1.60 x 0.90	2.54 x 1.37	2.50 x 1.37
examination time	5 min 40 sec	5 min 28 sec	5 min 40 sec	5 min 48 sec
time to repeat (TR)	814 ms	5948 ms	814 ms	5690 ms
time to echo (TE)	17 ms	102 ms	17 ms	102 ms
slice number	22	22	22	22
slice thickness	4 mm	4 mm	5 mm	5 mm
acquisition	coronary, axial	coronary	coronary, axial	coronary
distance factor	0.50	0.50	1.00	1.00
matrix size	138 x 256 (54%)	126 x 256 (56%)	138 x 256 (54%)	140 x 256 (55%)
field of view	230 mm	230 mm	350 mm	350 mm

body coil (for the 6 month old pigs) were used as receivers and positioned over the head and neck. Figure 2 shows the positioning of the pig and exemplarily the two acquisition directions (coronary and axial) of the sequences used.

#### Image evaluation

The magnetic resonance images were evaluated using the Able 3D-Doctor® Software (Able Software Corp., Lexington, MA, USA). A regional analysis was performed to evaluate the volume of the regions with increased signal intensity at the vaccination site (VS; left neck side) and at the control site (CS; right neck side). A region of interest (ROI; see white box in Fig. 3) was cre-

ated in order to cover the largest extent of the area with increased signal intensity at the VS. Regions with increased signal intensity within the ROI were defined by using the interactive segmentation function, which segments regions into different tissue classes by separating them according to defined signal intensities on a grey scale level from 0 (black) - 4096 (white). Regions with increased signal intensities or grey values (close to white) were classified as hyperintense regions. Within the same image the ROI of the VS was mirrored to the CS and the interactive segmentation function was applied using the same thresholds for signal intensity as on the VS (see Fig. 3). Five images from each sequence – starting at the injection point – were evaluated accordingly.



**Fig. 3: Exposure of the MR image evaluation using the interactive segmentation function of the Able 3D-Doctor Software®, exemplary for a T2-weighted coronary MR image**

VS represents the vaccination site and CS represents the control side. (a) T2-weighted coronary MR image, representing a region with increased signal intensity at the VS. (b) Same MR image with defined ROI (region of interest, white box) around the largest extent of the area with increased signal intensity. The area with increased signal is bordered using the interactive segmentation function. (c) The ROI is flipped to the CS and regions with increased signal and the same thresholds as the VS are bordered, if existing.

### Statistical analysis

The statistical software package SAS 9.3 (SAS Institute Inc., Cary, North Carolina, USA) was used for a variance analysis procedure, performing an F-test followed by a paired t-test. Thus differences between hyperintense volumes of the VS and the corresponding CS could be obtained for each sequence and vaccination group separately. The significance level was set at  $p < 0.05$ .

We used the matched paired t-test because the animals served both as control and as treatment groups at the same time. As described, one body side served as vaccination site and the opposite body side as control site (without vaccination). Therefore, we were dealing with dependent means (standard deviations) derived from volume calculations for voxels with increased signal intensities in the region of interest of the vaccination site and the

mirrored region of interest of the untreated control (body) side. The region of interest was mirrored along the (neck) spine.

### 3 Results

The results of the F-test and the paired t-test show obvious volume differences regarding VS and CS (see Vol\_diff, Tab. 4). These differences are significant ( $p < 0.05$ ) – in some cases highly significant ( $p < 0.001$ ) – in all used sequences (T1<sub>c</sub>, T2<sub>c</sub>) on the first day of examination. During follow-up examinations (day 3–29) volume differences between VS and CS decreased. In some examination groups significant volume differences could still be detected at the last day of examination (group III: T2<sub>c</sub>; group IV: T1<sub>c</sub>, T2<sub>c</sub>).

**Tab. 4: Results of the paired t-test to identify volume differences between the vaccination and the control site**

group	day	T1-weighted coronary MRI sequence (T1 <sub>c</sub> )		T2-weighted coronary MRI sequence (T2 <sub>c</sub> )	
		Vol_diff ± SD (cm <sup>3</sup> )	p-value	Vol_diff ± SD (cm <sup>3</sup> )	p-value
<b>I</b> (GnRF)	1	3.53 ± 0.86	< 0.01	7.78 ± 1.15	< 0.0001
	3	1.88 ± 0.58	< 0.01	1.92 ± 0.51	< 0.01
	8	2.21 ± 0.75	< 0.01	2.02 ± 0.54	< 0.01
	15	1.39 ± 0.42	< 0.01	0.15 ± 0.10	n.s.
	29	1.33 ± 0.98	n.s.	-0.74 ± 0.35	< 0.05
<b>II</b> (Parvovirus)	1	0.46 ± 0.14	< 0.01	0.28 ± 0.11	< 0.05
	3	0.71 ± 0.22	< 0.01	0.29 ± 0.11	< 0.05
	8	0.58 ± 0.14	< 0.001	0.12 ± 0.07	n.s.
	15	0.09 ± 0.10	n.s.	0.03 ± 0.04	n.s.
	22	0.13 ± 0.11	n.s.	-0.02 ± 0.07	n.s.
	29	0.12 ± 0.13	n.s.	0.11 ± 0.08	n.s.
<b>III</b> (Parvovirus + E. r. I)	1	0.77 ± 0.25	< 0.01	2.38 ± 0.71	< 0.01
	3	0.84 ± 0.30	< 0.05	2.44 ± 0.60	< 0.01
	8	0.47 ± 0.13	< 0.01	2.55 ± 0.60	< 0.001
	15	0.03 ± 0.13	n.s.	2.22 ± 0.54	< 0.001
<b>IV</b> (Parvovirus + E. r. II)	1	1.31 ± 0.39	< 0.01	1.52 ± 0.52	< 0.05
	3	2.83 ± 0.60	< 0.001	3.10 ± 1.61	n.s.
	8	2.71 ± 0.64	< 0.001	1.25 ± 0.97	n.s.
	15	1.56 ± 0.47	< 0.01	1.60 ± 0.46	< 0.01
	22	1.02 ± 0.45	< 0.05	2.17 ± 0.55	< 0.01

Because of technical problems no data could be obtained for group I on day 22. Vol\_diff describes the volume difference for the hyperintense volume at VS and CS. All arithmetic mean volume differences are specified with the corresponding standard deviations (SD) and the corresponding result of the paired t-test as p-value. n.s. = not significant ( $p > 0.05$ ). E. r. = *Erysipelothrix rhusiopathiae*.





The evaluated volume differences (Vol\_diff) differed between the groups on the same examination day. The highest volume differences between VS and CS were found in examination group I, no matter which sequence was used; the smallest volume differences were found in examination group II.

When we compare T1<sub>c</sub> and T2<sub>c</sub>, the evaluated volume differences of the four examination groups change as follows (on examination day 1): for group I the volume differences were doubled, for group II the evaluated mean volume differences were halved, for group III the volume differences were triplicated and for group IV a small increase of the mean volume differences was observed.

#### 4 Discussion and conclusions

The results of this study showed in each examination group – and therefore for every vaccine used – significant differences between VS and CS. These differences were significant on the first examination days for all groups, independent of the measurement sequence. As the anesthesia was injected into the muscles of the hind leg (*M. semitendinosus* / *M. semimembranosus*) and changes in the grey scales of the neck muscle tissue only can be detected at the VS, it is clear that (1) interactions of the vaccine with other injections can be excluded and that (2) the hyperintense region inside the muscle tissue of VS represents the local reaction caused by the injected vaccine. These results show that MRI allows detection and evaluation of three-dimensional local reactions within muscular and subcutaneous tissues caused by the injection of vaccines. These data, obtained in pigs, are in line with results of human studies, where MRI has been used to detect and monitor muscular damage (Fisher et al., 1986; Lovitt et al., 2006; Messineo et al., 1998; Schrank et al., 2005).

Additionally, this study showed that repeated MRI examinations of the same animal allow description of the progress of local reactogenicity individually for each animal. For example, group I showed (on day 1 for sequence T1<sub>c</sub>) an average volume difference of  $3.53 \pm 0.86 \text{ cm}^3$ , which decreased until day 29 to an average volume difference of  $1.33 \pm 0.98 \text{ cm}^3$  (see Tab. 4). In comparison, histopathologic examinations of animals, which are currently used in safety testing, offer only single snap shots of individual animals. The possibility to evaluate local reactions using follow-up examinations in living pigs by MRI – like it is used in humans – means that the animals do not have to be killed to identify possible reactions after vaccination by a *post mortem* examination. Animals can stay in the clinical trial until no further reaction is visible in MR images or until the reaction has reached an extent that compromises the animal's well-being. In conclusion, the use of MRI in clinical safety studies can contribute to reliable and well-documented safety data and allows an overall reduction of the number of animals needed to perform such trials.

Furthermore, this study also showed that local reactogenicity varies between groups and therefore between vaccines (see Tab. 4). For example, the mean volume difference of group I decreased continuously from examination day 1 to day 29,

whereas the mean volume difference of group IV increased on the first examination days and decreased from day 15 on (see Tab. 4). That shows that MRI can follow different progressions of local reactions initiated by different vaccines.

Additionally differences regarding the evaluated volume differences between VS and CS were found in this study (Tab. 4). That means that MRI can be used to rank vaccines regarding their tissue compatibility and this information can lead to a risk-benefit-assessment in safety testing of veterinary vaccines. This data can further be used to formulate recommendations on the animal welfare impact of vaccination – as massive local reactions at the injection site do also affect animal welfare.

It could be postulated that the volume differences between VS and CS could be solely caused by a volume effect of the injected volume, as volume effects were reported in the literature (Sidell et al., 1974; Steiness et al., 1974). As no control injection of saline solution was examined during this study, the volume effect cannot be totally excluded in this case. Earlier studies, however, have not reported pathologic conditions after administration of 2 ml of saline solution in pigs (Steiness et al., 1978). However, the injection volume in this study was 2 ml for all examination groups. If the volume effect were the only reason for the alterations in the MR images and the corresponding volume differences, the volume differences should be comparable for all examination and age groups. As shown in the results (Tab. 4), the volume differences differed among the examination groups (I-IV) and among the age groups (12 weeks old and 6 month old). The true volume effect cannot exceed the detected volume difference for group II, which was the smallest difference between injection and control site (Tab. 4). Therefore, these study results show that different volume differences are caused mainly by different vaccine ingredients. Further studies could evaluate a possible volume effect by injecting saline solution in different age groups as a control compared to vaccine injections.

In human studies, MRI is used to detect alterations in muscle tissue (Lovitt et al., 2006; May et al., 2000; Schrank et al., 2005). By choosing different sequence parameters for MR imaging, a multitude of pathologic conditions can be illustrated (Berquist et al., 1985; Hodgson, 2010). In order to use this potential for this study, two different MRI sequence parameters (T1- and T2-weighted) were used (see Tab. 3). Different pathologic conditions are described in the literature like hematoma, edema, fatty infiltration or inflammation, which result in alterations in tissue parameters and therefore in an illustration in T1- or T2-weighted MR images. These pathologic conditions result in areas with increased signal intensity in T1- and T2-weighted MR images (Lovitt et al., 2006; May et al., 2000; Schrank et al., 2005). In this study image evaluation was performed by evaluating volumes of regions with hyperintense signals at the VS and CS (see Fig. 3). These volumes differed between the different used sequences showing different pathologic reactions as described in the literature (Lovitt et al., 2006; May et al., 2000; Schrank et al., 2005):

##### 1. T2-weighted MR images:

Regions with hyperintense signals in the T2-weighted MR images represent edematous tissue or fatty infiltration (May et al.,

2000; Schrank et al., 2005; Shellock et al., 1996). Fat tissue and especially fatty infiltration on both neck sides (VS and CS) is normally spread evenly, if no injection is given. That means that in this study all hyperintense regions inside VS – which were not detected in CS – represent edematous tissue (see Fig. 3). Such tissue regions were found in all examination groups and were significant on day 1 in all groups (see Tab. 4). The detected volume differences varied depending on the vaccine used. In group I, a volume difference of  $7.78 \pm 1.15 \text{ cm}^3$  was detected on day 1 (T2c), the smallest edematous volume difference ( $0.28 \pm 0.11 \text{ cm}^3$ , day 1) was found in group II. Additionally, the volumes of the edematous tissue in this study differed in their progression. For group IV, the edematous volume difference increased until day 3 and then decreased. For group I a continuous decrease in volume difference was detected. These variations seem to be caused by the effective parts of the different vaccines (i.e., adjuvants, preservatives or other ingredients). The ingredients seem to determine the extent and duration of an edematous reaction. Each used vaccine in this study differed in its composition. This assumption is confirmed by human studies which displayed different local reactions caused by different adjuvants (Batista-Duharte et al., 2013; Edelman, 1980; Gupta et al., 1993).

In group III and IV, significant differences were also found on the last day of examination. These could be caused by fibrotic regeneration of degenerated muscle fibers (Serrano and Muñoz-Cánoves, 2010).

## 2. T1-weighted MR images:

Regions with hyperintense signals inside the T1-weighted MR images can represent inflammatory tissue, fatty infiltration or hematoma (May et al., 2000; Schrank et al., 2005). As explained before, during this study the anesthesia injections were given into the ham muscle in order to avoid interdependencies. In case of fat tissue or especially fatty infiltration, the hyperintense areas would be spread evenly on both neck sides (VS and CS), if apparent. Therefore, all regions with hyperintense signal inside T1-weighted images can be backtracked to inflammatory reactions due to the vaccination.

In conclusion, this study demonstrates that MRI, as a non-invasive tool, is suitable to detect, monitor and evaluate local reactions in clinical safety tests of pig vaccines. This method has the potential to reduce the number of animals used in safety tests for veterinary vaccines. Animals can be monitored individually at several time points throughout the study. The image evaluation, performed by defining a ROI around the largest extent of the local reaction and evaluating the volume of hyperintense regions, appears to be a reliable method. Using appropriate software, this evaluation method documents the three-dimensional extent of a vaccination-induced local reaction. These volume data can easily be transferred to other treatments like any kind of medicines, other compositions, other animals or other examination days. This study in pigs documents remarkable differences between the vaccines used, with regard to evaluated volumes, extent and duration of local reactions. Additional studies with younger pigs would cover the whole spectrum of age categories. The development of a scoring system to docu-

ment the extent and the severity of a local reaction may also be a helpful tool to standardize safety evaluations of injectable veterinary medicines, especially vaccines.

To sum up, the use of MRI in clinical safety studies offers reliable and well-documented safety data, which could allow an overall reduction of the number of animals needed to perform such trials.

A proof of principle is necessary to establish this method as a standard tool for safety testing.

## References

- Batista-Duharte, A., Portuondo, D., Carlos, I. Z. and Pérez, O. (2013). An approach to local immunotoxicity induced by adjuvanted vaccines. *Int Immunopharmacol* 17, 526-536. <http://dx.doi.org/10.1016/j.intimp.2013.07.025>
- Berquist, T. H., Brown, M. L., Fitzgerald, R. H. and May, G. R. (1985). Magnetic resonance imaging: Application in musculoskeletal infection. *Magn Reson Imaging* 3, 219-230. [http://dx.doi.org/10.1016/0730-725X\(85\)90350-9](http://dx.doi.org/10.1016/0730-725X(85)90350-9)
- EC (2001). Directive 2001/82/EC of the European Parliament and of the Council of 6 November 2001 on the Community code relating to veterinary medicinal products. ANNEX I: Requirements and analytical protocol, safety tests, pre-clinical and clinical for tests of veterinary medicinal products. TITLE II: Requirements for immunological veterinary medicinal products. PART 7: Safety testing.
- Edelman, R. (1980). Vaccine adjuvants. *Rev Infect Dis* 2, 370-383. <http://dx.doi.org/10.1093/clinids/2.3.370>
- EDQM – European Directorate for the Quality of Medicines and Health Care (2008). *European Pharmacopoeia (Ph. Eur.)*. 6<sup>th</sup> edition. Volume 1, Chapter 5.2.6. Evaluation of safety of veterinary vaccines and immunosera (p 536 ff.).
- Faul, F., Erdfelder, E., Lang, A.-G. and Buchner, A. (2007). G\*Power 3: A flexible statistical power analysis program for the social, behavioral, and biomedical sciences. *Behav Res Methods* 39, 175-191. <http://dx.doi.org/10.3758/BF03193146>
- Faul, F., Erdfelder, E., Buchner, A. and Lang, A.-G. (2009). Statistical power analyses using G\*Power 3.1: Tests for correlation and regression analyses. *Behav Res Methods* 41, 1149-1160. <http://dx.doi.org/10.3758/BRM.41.4.1149>
- Fisher, M. R., Dooks, G. C., Hricak, H. et al. (1986). Magnetic resonance imaging of the normal and pathologic muscular system. *Magn Reson Imaging* 4, 491-496. [http://dx.doi.org/10.1016/0730-725X\(86\)90029-9](http://dx.doi.org/10.1016/0730-725X(86)90029-9)
- German Federal Ministry for Food, Agriculture and Consumer Protection (2009). Verordnung zum Schutz landwirtschaftlicher Nutztiere und anderer zur Erzeugung tierischer Produkte gehaltener Tiere bei ihrer Haltung (TierSchNutzV) in der Fassung der Bekanntmachung vom 22. August 2006 (BGBl. I S. 2043), die durch Artikel 1 der Verordnung vom 1. Oktober 2009 (BGBl. I S. 3223) geändert worden ist. Neugefasst durch Bek. v. 22.8.2006 I 2043; geändert durch Art. 1 V v. 1.10.2009 I 3223.
- Germany (2013). Tierschutzgesetz in der Fassung der Bekanntmachung vom 18. Mai 2006 (BGBl. I S. 1206, 1313), das



- durch Artikel 4 Absatz 90 des Gesetzes vom 7. August 2013 (BGBl. I S. 3154) geändert worden ist. Neugefasst durch Bek. v. 18.5.2006 I 1206, 1313; zuletzt geändert durch Art. 1 G v. 4.7.2013 I 2182.
- Gupta, R. K., Relyveld, E. H., Lindblad, E. B. et al. (1993). Adjuvants – a balance between toxicity and adjuvanticity. *Vaccine* 11, 293-306. [http://dx.doi.org/10.1016/0264-410X\(93\)90190-9](http://dx.doi.org/10.1016/0264-410X(93)90190-9)
- Hodgson, R. J. (2010). The basic science of MRI. *Orthop Trauma* 25, 119-130. <http://dx.doi.org/10.1016/j.morth.2010.12.002>
- Kilkenny C., Browne, W. J., Cuthill, I. C. et al. (2010). Improving bioscience research reporting: The ARRIVE guidelines for reporting animal research. *PLoS Biol* 8, e1000412. <http://dx.doi.org/10.1371/journal.pbio.1000412>
- Kuo, G. P. and Carrino, J. A. (2007). Skeletal muscle imaging and inflammatory myopathies. *Curr Opin Rheumatol* 19, 530-535. <http://dx.doi.org/10.1097/BOR.0b013e3282efdc66>
- Lovitt, S., Moore, S. L. and Marden, F. A. (2006). The use of MRI in the evaluation of myopathy. *Clin Neurophysiol* 117, 486-495. <http://dx.doi.org/10.1016/j.clinph.2005.10.010>
- May, D. A., Disler, D. G., Jones, E. A. et al. (2000). Abnormal signal intensity in skeletal muscle at MR Imaging: Patterns, pearls, and pitfalls. *Radiographics* 20, 295-315. [http://dx.doi.org/10.1148/radiographics.20.suppl\\_1.g00oc18s295](http://dx.doi.org/10.1148/radiographics.20.suppl_1.g00oc18s295)
- Messineo, D., Cremona, A., Trinci, M. et al. (1998). MRI in the study of distal primary myopathies and of muscular alterations due to peripheral neuropathies: Possible diagnostic capacities of MR equipment with low intensity field (0.2 T) dedicated to peripheral limbs. *Magn Reson Imaging* 16, 731-741. [http://dx.doi.org/10.1016/S0730-725X\(98\)00080-0](http://dx.doi.org/10.1016/S0730-725X(98)00080-0)
- Nägele, M. and Hahn, D. (1990). Kernspintomographie. In D. E. Pongratz, C. D. Reimers, D. Hahn et al. (ed.), *Atlas der Muskelkrankheiten/Pongratz* (25-29). Kösel, Kempten, Germany: Urban & Schwarzenberg. ISBN 3-541-13021-0
- Pipe, J. G. (1999). Basic spin physics. *Magn Reson Imaging Clin N Am* 7, 607-27.
- Reimers, C. D. (1990). Vergleich der bildgebenden Verfahren. In D. E. Pongratz, C. D. Reimers, D. Hahn et al. (ed.), *Atlas der Muskelkrankheiten/Pongratz* (34). Kösel, Kempten, Germany: Urban & Schwarzenberg. ISBN 3-541-13021-0
- Russel, W. M. S. and Burch, R. L. (1959). *The Principles of Humane Experimental Technique*. London: Methuen. Reprinted by Universities Federation for Animal Welfare, Potters Bar, UK, 1992. [http://altweb.jhsph.edu/pubs/books/humane\\_exp/het-toc](http://altweb.jhsph.edu/pubs/books/humane_exp/het-toc).
- Schedel, H., Reimers, C. D., Nägele, M. et al. (1992). Imaging techniques in myotonic dystrophy. A comparative study of ultrasound, computed tomography and magnetic resonance imaging of skeletal muscles. *Eur J Radiol* 15, 230-238. [http://dx.doi.org/10.1016/0720-048X\(92\)90113-N](http://dx.doi.org/10.1016/0720-048X(92)90113-N)
- Schrank, B., Urban, P. and Lörcher, U. (2005). Der Einsatz der Magnetresonanztomographie der Muskulatur bei der Diagnose neuromuskulärer Erkrankungen. *Klin Neuroradiol* 15, 241-255. <http://dx.doi.org/10.1007/s00062-005-6419-1>
- Serrano, A. L. and Muñoz-Cánoves, P. (2010). Regulation and dyregulatopn of fibrosis in skeletal muscle. *Exp Cell Res* 316, 3050-3058. <http://dx.doi.org/10.1016/j.yexcr.2010.05.035>
- Shellock, F. G., Tyson, L. L. and Fleckenstein, J. L. (1996). Diagnostic imaging of skeletal muscle exercise physiology and pathophysiology. In J. L. Fleckenstein, J. V. Crues and C. D. Reimers (ed.), *Muscle Imaging in Health and Disease*. Springer-Verlag New York, Inc. [http://dx.doi.org/10.1007/978-1-4612-2314-6\\_7](http://dx.doi.org/10.1007/978-1-4612-2314-6_7)
- Sidell, F. R., Culver, D. L. and Kaminskis, A. (1974). Serum creatine phosphokinase activity after intramuscular injection. The effect of dose, concentration, and volume. *J Am Med Assoc* 229, 1894-1897. <http://dx.doi.org/10.1001/jama.1974.03230520036027>
- Steiness, E., Svendsen, O. and Rasmussen, F. (1974). Plasma digoxin after parenteral administration. Local reaction after intramuscular injection. *Clin Pharmacol Ther* 16, 430-434.
- Steiness, E., Rasmussen, F., Svendsen, O. and Nielsen, P. (1978). A comparative study of serum creatine phosphokinase (CPK) activity in rabbits, pigs and humans after intramuscular injection of local damaging drugs. *Acta Pharmacol Toxicol* 42, 357-364. <http://dx.doi.org/10.1111/j.1600-0773.1978.tb02217.x>
- Stokes, W. S., Kulpa-Eddy, J. and McFarland, R. (2011). The international workshop on alternative methods to reduce, refine, and replace the use of animals in vaccine potency and safety testing: Introduction and summary. *Procedia In Vaccinology* 5, 1-15. <http://dx.doi.org/10.1016/j.provac.2011.10.001>
- Tanttu, J. and Sepponen R. E. (1996). Basic principles of magnetic resonance imaging. In J. L. Fleckenstein, J. V. Crues and C. D. Reimers (ed.), *Muscle Imaging in Health and Disease* (21-39). Springer Verlag. ISBN 0-387-94231-9
- Walker, U. A. (2008). Imaging tools for the clinical assessment of idiopathic inflammatory myositis. *Curr Opin Rheumatol* 20, 656-661. <http://dx.doi.org/10.1097/BOR.0b013e3283118711>

### Conflict of interest statement

No author has potential conflicts of interest.

### Acknowledgement

This research project was funded in total by the German Federal Ministry of Education and Research (BMBF project number 0316009B).

### Correspondence to

Maren Bernau, Dr. med. vet.  
Livestock Center Oberschleissheim  
Veterinary Faculty of the Ludwig-Maximilians-University Munich  
St. Hubertusstrasse 12  
85764 Oberschleissheim  
Germany  
Phone: 0049 89 2180 76061  
e-mail: Maren.Bernau@lmu.de